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McGahon, M. K., Fernández, J. A., Dash, D. P., McKee, J., Simpson, D. A., Zholos, A. V., McGeown, J. G., & Curtis, T. M. (2016). TRPV2 Channels Contribute to Stretch-Activated Cation Currents and Myogenic Constriction in Retinal Arterioles. *Investigative ophthalmology & visual science*, 57(13), 5637-5647. <https://doi.org/10.1167/iovs.16-20279>

Published in:

Investigative ophthalmology & visual science

Document Version:

Publisher's PDF, also known as Version of record

Queen's University Belfast - Research Portal:

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TRPV2 Channels Contribute to Stretch-Activated Cation Currents and Myogenic Constriction in Retinal Arterioles

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Submitted: July 7, 2016

Accepted: September 4, 2016

Citation: McGahon MK, Fernández JA, Dash DP, et al. TRPV2 channels contribute to stretch-activated cation currents and myogenic constriction in retinal arterioles. *Invest Ophthalmol Vis Sci.* 2016;57:5637–5647. DOI: 10.1167/iovs.16-20279

PURPOSE. Activation of the transient receptor potential channels, TRPC6, TRPM4, and TRPP1 (PKD2), has been shown to contribute to the myogenic constriction of cerebral arteries. In the present study we sought to determine the potential role of various mechanosensitive TRP channels to myogenic signaling in arterioles of the rat retina.

METHODS. Rat retinal arterioles were isolated for RT-PCR, Fura-2 Ca^{2+} microfluorimetry, patch-clamp electrophysiology, and pressure myography studies. In some experiments, confocal immunolabeling of wholemount preparations was used to examine the localization of specific mechanosensitive TRP channels in retinal vascular smooth muscle cells (VSMCs).

RESULTS. Reverse transcription-polymerase chain reaction analysis demonstrated mRNA expression for TRPC1, M7, V1, V2, V4, and P1, but not TRPC6 or M4, in isolated retinal arterioles. Immunolabeling revealed plasma membrane, cytosolic and nuclear expression of TRPC1, M7, V1, V2, V4, and P1 in retinal VSMCs. Hypoosmotic stretch-induced Ca^{2+} influx in retinal VSMCs was reversed by the TRPV2 inhibitor tranilast and the nonselective TRPP1/V2 antagonist amiloride. Inhibitors of TRPC1, M7, V1, and V4 had no effect. Hypoosmotic stretch-activated cation currents were similar in Na^+ and Cs^+ containing solutions suggesting no contribution by TRPP1 channels. Direct plasma membrane stretch triggered cation current activity that was blocked by tranilast and specific TRPV2 pore-blocking antibodies and mimicked by the TRPV2 activator, Δ^9 -tetrahydrocannabinol. Preincubation of retinal arterioles with TRPV2 blocking antibodies prevented the development of myogenic tone.

CONCLUSIONS. Our results suggest that retinal VSMCs express a range of mechanosensitive TRP channels, but only TRPV2 appears to contribute to myogenic signaling in this vascular bed.

Keywords: TRPV2, myogenic tone, blood flow autoregulation, retinal arterioles, ion channels

In common with many organs, blood flow in the retina is kept relatively uniform even during large changes in perfusion pressure.¹ This autoregulation of blood flow protects the capillary network from excessive pressure and ensures that oxygen delivery and waste removal is maintained at a constant level, despite variations in mean arterial pressure.² The myogenic response of small resistance arteries and arterioles is considered to be one of the primary physiological mechanisms underlying blood flow autoregulation.^{3,4} The myogenic response refers to the inherent ability of blood vessels to constrict when intravascular pressure increases, and conversely, to dilate when it decreases.⁵ Previous work has shown that the myogenic response is independent of the vascular endothelium and mediated by the reaction of the vascular smooth muscle cells (VSMCs) in the vessel wall.^{3,6} Despite its importance in blood flow autoregulation, the exact cellular and molecular mechanisms underlying this response have yet to be fully elucidated.

Unravelling the mechanisms responsible for the myogenic response requires an appreciation of how the VSMCs detect and respond to changes in intraluminal pressure. Current evidence suggests that the mechanical stretch generated by increased intravascular pressure activates cation channels on the surface of the VSMCs.^{7,8} This causes cell membrane

potential depolarization and an increase in voltage-dependent Ca^{2+} influx, leading to contraction.^{9–12} However, whilst numerous studies have demonstrated the presence of stretch-activated cation currents in VSMCs,^{13–15} our knowledge of the molecular identity of the underlying channels remains incomplete. Transient receptor potential (TRP) channels represent good candidates for mediating stretch-activated cation currents and pressure-induced depolarization in VSMCs.^{7,8} In mammals, TRP channel proteins form a cation-permeable ion channel family with 28 members, which may be grouped into six subfamilies on the basis of amino acid sequence homology (TRPC, TRPV, TRPM, TRPA, TRPP, and TRPML¹⁶). Although TRP channels display a remarkable assortment of activation mechanisms (e.g., heat, lipids, protons), recent studies suggest a general role for these channels in mechanosensation.¹⁷ Indeed, TRPC1,5,6, TRPM4,7, TRPV1,2,4, and TRPP1 (PKD2) have all been detected in VSMCs and implicated in mechanotransduction.¹⁸ Additionally, TRPA1 has been proposed to be a component of the mechanosensitive transduction channel of vertebrate hair cells,¹⁹ but has yet to be detected in VSMCs.²⁰ Recent studies have shown that TRPC6, TRPM4, and TRPP1 channels contribute to pressure-induced depolarization and myogenic vasoconstriction in arteries of the cerebral circulation.^{7,8,21} However, the role of TRP channels in myogenic



TABLE 1. Inhibitors and Experimental Approaches Used to Identify the Involvement of Specific MechanoTRP Channels in Hypotonic Stretch-Induced $[Ca^{2+}]_i$ Responses

Inhibitor/Antibody	Dilution/Concentration	Experimental Approach	MechanoTRP Channel Target
T1E3 Ab	1:200	Preincubation, 2 h at 21°C	TRPC1 ²⁸
TRPC1 Alo Ab	1:200	Preincubation, 2 h at 21°C	TRPC1 ²⁹
FTY720	1 μ M	Applied during plateau of response	TRPM7 ³⁰
Amiloride	100 μ M	Applied 30 s before and throughout second response	TRPP1/V2 ^{31,32}
Capsazepine	5 μ M	Applied during plateau of response	TRPV1 ^{33,34}
Tranilast	100 μ M	Applied during plateau of response	TRPV2 ^{35,36}
HC067047	1 μ M	Applied during plateau of response	TRPV4 ^{37,38}

signaling in other vascular beds, including the retina, remains less clear. In aortic myocytes, TRPV2 has been shown to contribute to nonselective cation currents evoked by hypotonic cell swelling.²² These results suggest that TRPV2 may function as a mechanosensitive channel in VSMCs, but its involvement in myogenic vasoregulation has yet to be tested.

In the present study, we evaluate the potential contribution of various mechanosensitive TRP (mechanoTRP) channels to myogenic signaling in intact retinal arterioles. We show that although retinal VSMCs express a range of putative mechanoTRP channel proteins, only TRPV2 appears to contribute to stretch-evoked cation currents and myogenic signaling in this vascular bed.

MATERIALS AND METHODS

For detailed experimental procedures, see Supplementary material online.

Animals

Retinas and retinal arterioles used for these studies were isolated from male Sprague-Dawley rats (200–350 g; Harlan, Bicester, UK). Animal use conformed to the standards in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were euthanized by CO₂ and all experiments undertaken in accordance with EU guidelines (Directive 210/63/EU).

Retinal Arteriole Isolation

Retinal arterioles were isolated as previously described.^{23,24} Experiments were carried out within 8 hours of vessel isolation.

RT-PCR

To assess mechanoTRP channel mRNA expression, isolated retinal arterioles were collected under microscopic magnification and transferred into total RNA extraction buffer (RNeasy Mini Kit; Qiagen, Crawley, UK). Reverse transcription-polymerase chain reaction was carried out according to previously established protocols within our laboratory^{24,25} using primers for specific mechanoTRP channels of interest (Supplementary Table S1).

Immunohistochemistry

Immunohistochemistry was performed on retinal arterioles still embedded within retinal wholemount preparations as previously described.^{25,26} Fixed tissue was probed with relevant anti-TRP channel (Supplementary Table S2) and anti- α -smooth muscle actin (α SMA) antibodies (Abcam, Cambridge, UK) for 3 days at 4°C. Nuclei were labelled using TO-PRO-3 (Life

Technologies, Paisley, UK). Primary antibodies were detected using appropriate fluorophore-conjugated secondary antibodies and images acquired using a Leica SP5 laser scanning confocal microscope. Following image capture, retinal arterioles were digitally segmented from the surrounding retinal neuropil using the α SMA channel.

Intracellular Ca²⁺ Recording

Ca²⁺ imaging experiments were performed as previously described.^{23–25} Briefly, isolated retinal arterioles were loaded with 5 μ M fura-2AM (Abcam) for 2 hours at room temperature. This exclusively loads the VSMC layer of these vessels.²³ Following loading, vessels were intermittently excited with ultraviolet light at 340 and 380 nm (5 Hz) and emissions at 510 nm recorded using a photomultiplier tube. Arterioles were exposed to hypotonic solution (Supplementary Table S3) to stretch the cells and various inhibitors used to identify the involvement of specific mechanoTRP channels in mediating the associated $[Ca^{2+}]_i$ responses (Table 1). Background fluorescence was determined by quenching with 10 mM MnCl₂. Background-corrected fluorescence ratios (F₃₄₀/F₃₈₀) were converted to $[Ca^{2+}]_i$ using the Grynkiewicz equation.²⁷

Patch-Clamp Recording

Whole-cell and cell-attached patch clamp recordings were made from retinal VSMCs whilst still embedded within their parental arterioles.^{25,26} Vascular smooth muscle cells were electrically uncoupled from their neighboring cells by enzymatic digestion.^{26,39} Currents were recorded using an Axopatch 200B amplifier (Molecular Devices, CA, USA) and pCLAMP10 was used for data acquisition and analysis. To isolate stretch-activated cation currents, pharmacological inhibitors of BK, K_v, L-type Ca²⁺, and TREK-1 channels were included in the external bathing medium (whole-cell recordings) or internal pipette solution (cell-attached recordings; see supplemental methods). Whole-cell recordings were performed using the perforated-patch configuration using amphotericin B (Supplementary Table S3) as the perforating agent. Whole-cell currents were elicited using voltage ramp protocols from –80 to +80 mV applied over 1 second from a holding potential of 0 mV. Cell-attached recordings were performed in high K⁺ solution to set the resting membrane potential toward 0 mV. To prevent contamination from Cl[–] currents, stretch-activated cation current activity was monitored at the Cl[–] equilibrium potential (+52 mV). To stretch the plasma membrane, negative pressure (–45 mm Hg) was applied through the recording electrode using a syringe connected to digital manometer.

Arteriolar Pressure Myography

Assessment of myogenic tone was performed as previously described.⁴⁰ Arteriolar segments were cannulated at one end with a glass micropipette and occluded at the opposite end

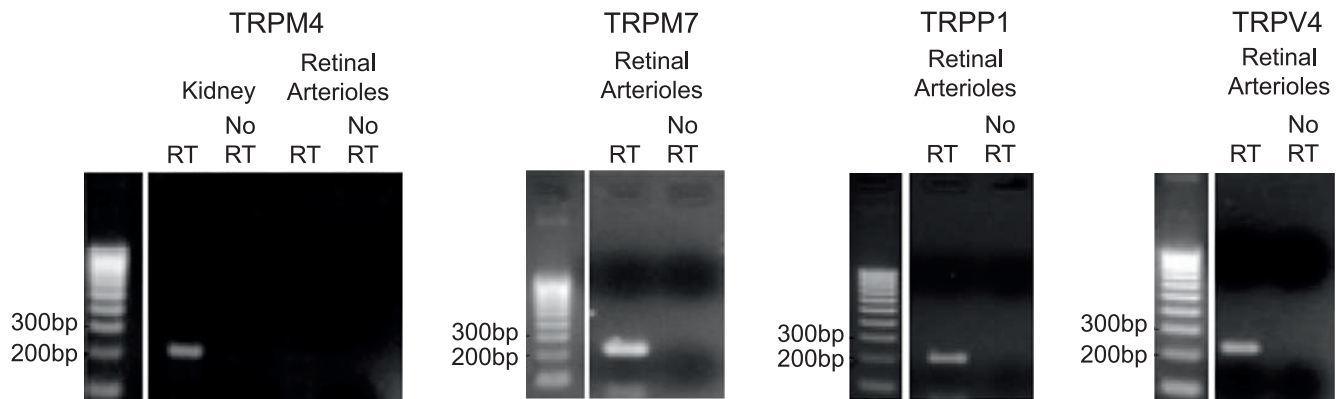


FIGURE 1. mRNA transcript expression of mechanoTRP channels in isolated arterioles from rat retina. Transcripts for TRPM7, TRPP1 (PKD2) and TRPV4 were detected; TRPM4 was absent.

using tungsten wire slips. Vessels were pressurized to 40 mm Hg and images captured using a MCN-BO13U microscope camera (Mightex, Pleasanton, CA, USA) at a rate of 140 frames per minute. Vessel diameter measurements were made using MyoTracker software.⁴¹ At the end of each experiment, the maximum (or passive) diameter was determined by exposing arterioles to wortmannin (10 μ M) in the presence of Ca^{2+} -free Hanks' solution.¹²

Data Analysis

All data are presented as means \pm SEM. Values of *n* refer to the numbers of arterioles for the Ca^{2+} imaging and vessel myography experiments or the number of cells for the patch-clamp studies. Data from at least four animals were obtained for each experimental protocol. Data were tested for normality and appropriate statistical tests applied as detailed in the online supplementary materials section. A *P*-value of 0.05 was considered statistically significant (* = *P* < 0.05, ** = *P* < 0.01, *** = *P* < 0.001). Statistical comparisons were performed using Excel or Prism 5 for Windows (Graphpad Software, Inc., CA, USA).

RESULTS

MechanoTRP Channel Expression and Localization in Retinal Arterioles

To investigate the potential role of specific mechanoTRP channels in the myogenic constriction of retinal arterioles, we

TABLE 2. Summary of mRNA Transcript Expression of MechanoTRP Channels in Retinal Arterioles

MechanoTRP Channel	Expression in Retinal Arterioles	Positive Control
TRPA1	—*	Brain*
TRPC1	+*	
TRPC5	—*	Brain*
TRPC6	—*	Brain*
TRPM4	—	Kidney
TRPM7	+	
TRPP1	+	
TRPV1	+*	
TRPV2	+*	
TRPV4	+	

* Detection of TRPC1, TRPV1, and TRPV2 and absence of TRPA1, TRPC5, and TRPC6 have been previously reported.²⁴

began by undertaking RT-PCR analysis of isolated rat retinal arteriole segments. We have previously demonstrated mRNA expression of TRPC1, TRPV1, and TRPV2 in these vessels, whilst transcripts for TRPC5, TRPC6, and TRPA1 could not be detected.²⁴ This work was extended to investigate the mRNA expression of other putative mechanoTRP channels, including TRPV4, TRPM4, TRPM7, and TRPP1 (PKD2). All of these channels were found to be expressed at the mRNA level in retinal arterioles with the exception of TRPM4 (Fig. 1; Table 2).

To confirm protein expression and to determine the cell-specific localization of those mechanoTRP channels found to be expressed at the mRNA level in retinal arterioles, we undertook confocal immunolabeling studies. Our primary focus was to identify those channels present on the VSMCs, the relevant cell type in the myogenic response. Retinal arterioles were immunolabeled whilst still embedded within retinal wholemount preparations and the VSMCs identified by staining for α SMA (Fig. 2Aii). α -Smooth muscle actin staining was absent at the border regions between adjacent VSMCs (Fig. 2Aii, arrows). Such regions correspond to the VSMC plasma membranes as they stained positively with the plasma membrane marker, isolectin B4 (Figs. 2Ai, 2Aiv). TRPC1 was found to be localized to VSMC plasma membranes, the cell cytoplasm, and to a lesser extent cell nuclei (as indicated by co-localization with TOPRO3 nuclear staining) in a pattern resembling the nucleoplasmic reticulum (Fig. 2B). TRPM7 was distributed in a punctate fashion throughout the VSMC layer, with some puncta localized to the plasma membrane (Fig. 2C). TRPP1 and TRPV1 were predominantly expressed in the cytosol of the VSMCs, with lower levels of expression detected at the plasma membrane and cell nuclei (Figs. 2D, 2E). Like TRPM7, TRPV2 was distributed in a diffuse punctate pattern throughout the VSMCs, although a larger proportion of the channels were observed to coincide with the plasma membrane (Fig. 2F). TRPV4 was found to co-localize mainly with the VSMC nuclei, with some punctate staining also observed at the plasma membrane and in cytosolic regions (Fig. 2G). Taken together, these results suggest that all of the mechanoTRP channels identified at the mRNA level in isolated retinal arterioles are expressed at the protein level in the VSMCs of these vessels and all showed some expression in the plasma membrane, consistent with a possible role in the generation of stretch-activated currents (Table 3).

MechanoTRPs and Hypotonic Stretch-Induced Ca^{2+} Influx and Cation Currents

To narrow down which of the mechanoTRP channels expressed in retinal arterioles might contribute functionally

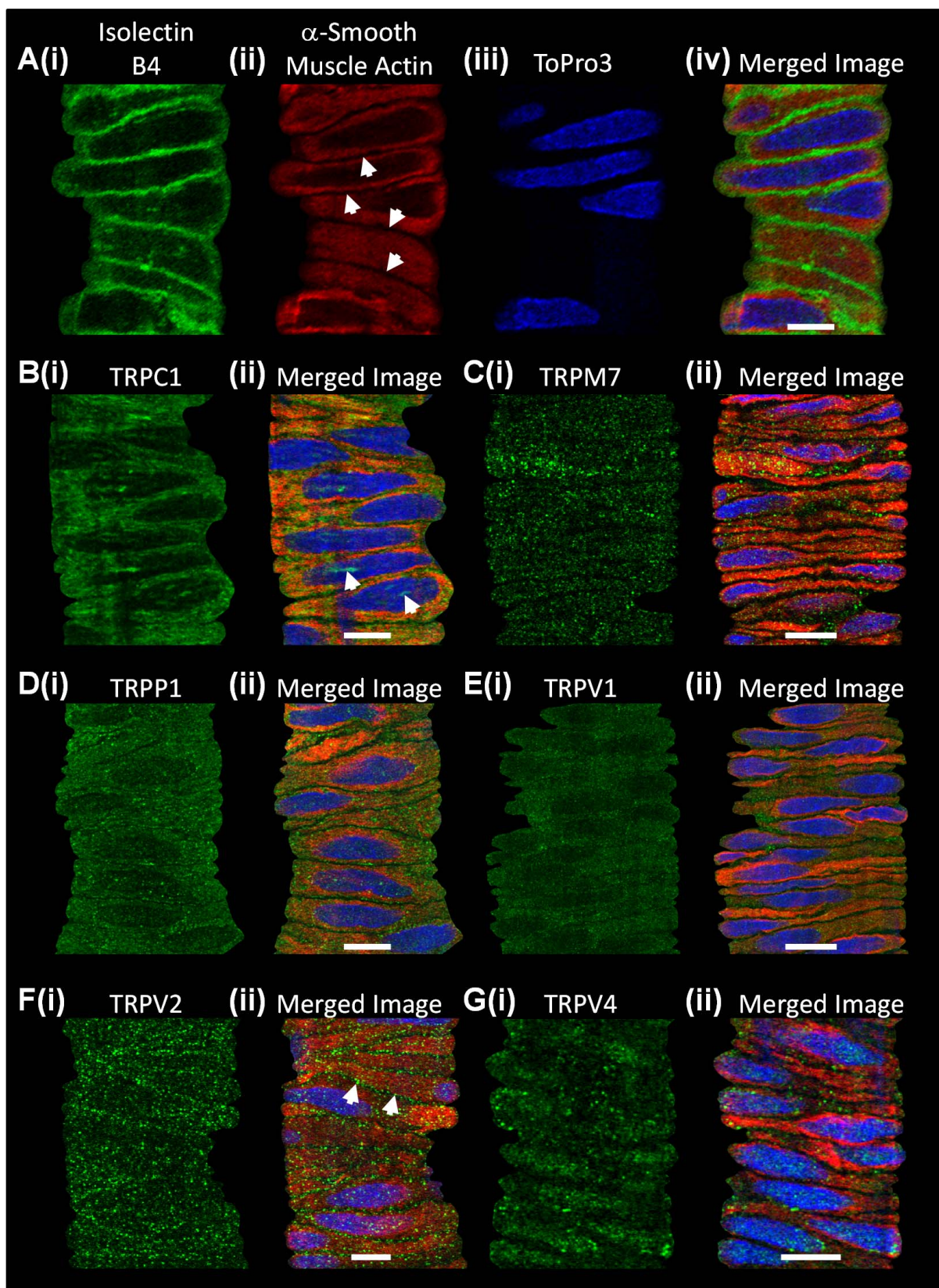


FIGURE 2. Immunolocalization of mechanoTRP channels in rat retinal VSMCs. (A) Confocal images of a rat retinal arteriole within a wholemount preparation labeled with Isolectin B4 (Ai, green; plasma membrane marker), α SMA antibody (Aii, red; smooth muscle cell marker), and TO-PRO3 (Aiii, pseudo-colored blue; nuclear marker). Images have been segmented on the basis of the α SMA channel to specifically isolate the blood vessel staining. Arrows in Aii denote plasma membrane regions between adjacent cells that were absent for α SMA, but positive for Isolectin B4 staining (Aiv, merged image). (B–G) Distribution patterns of TRPC1 (B), TRPM7 (C), TRPP1 (D), TRPV1 (E), TRPV2 (F), and TRPV4 (G) in VSMCs of retinal

arterioles. *Left hand panels (i)* show TRP channel immunolabeling segmented on the basis of the α SMA channel. *Right hand panels (ii)* show merged images of the TRP channel staining with α SMA (red) and TOPRO3 nuclear labelling (pseudo-colored blue). Arrows in **Bii** highlight TRPC1 nuclear co-localization in a pattern resembling the nucleoplasmic reticulum. Arrows in **Fii** denote strong immunolabeling of TRPV2 in plasma membrane regions of the VSMCs. Scale bars: 10 μ m.

to stretch-dependent myogenic vasoregulation, we investigated the involvement of each of these channels in mediating stretch-induced Ca^{2+} influx responses in retinal VSMCs. Vascular smooth muscle cells were loaded with fura-2 Ca^{2+} indicator dye and changes in $[\text{Ca}^{2+}]_i$ recorded following exposure to hypotonic bathing solution to cause cell swelling and stretching of the cell membrane. Reduction of the external osmolarity by 100 mOsm resulted in a gradual increase in $[\text{Ca}^{2+}]_i$ that plateaued within 1 to 2 minutes (Fig. 3Ai; Supplementary Fig. S1). This response was rapidly reversed by removal of extracellular Ca^{2+} (Figs. 3Ai, 3Aii), suggesting that it resulted primarily from Ca^{2+} influx across the VSMC membrane. Inhibitors of TRPC1, TRPM7, TRPV1, and TRPV4 had no effect on hypotonic stretch-induced $[\text{Ca}^{2+}]_i$ responses (Figs. 3B, 3C, 3E, 3G), while the nonselective TRPP1/V2 inhibitor, amiloride, and the TRPV2 inhibitor, tranilast, partially and fully inhibited the Ca^{2+} influx, respectively (Figs. 3D, 3F). These results suggest that TRPP1 and/or TRPV2 may be the main TRP channels underlying stretch-activated signaling in retinal VSMCs.

To investigate further the possible contribution of TRPV2 and TRPP1 channels to hypotonic stretch-induced responses in retinal VSMCs, whole-cell patch-clamp experiments were performed. In Na^+ containing external solution, application of hypotonic medium activated an inward current at -80 mV, which reversed to become an outward current (with slight outward rectification) at voltages positive to -3.8 ± 3.6 mV

(E_{rev} difference current; $n = 10$; Fig. 4A). Under these recording conditions, and based on the known permeability properties of the two channels,^{42–44} $E_{\text{rev}}(\text{TRPV2})$ and $E_{\text{rev}}(\text{TRPP1})$ were estimated to be -9.9 and -56.8 mV, respectively. These data suggest that TRPP1 channels are unlikely to contribute significantly to hypotonic stretch-induced cation currents in these cells. To test this further, we took advantage of known differences in the permeability properties of TRPV2 and TRPP1 channels. In particular, it has previously been shown that TRPV2 displays an approximate equal permeability to Na^+ and Cs^+ ions ($P_{\text{Cs}}/P_{\text{Na}} \approx 0.95^{42}$), whereas TRPP1 is virtually impermeable to Cs^+ ($P_{\text{Cs}}/P_{\text{Na}} \approx 0^{32,44}$). In Cs^+ containing external solution, application of hypotonic medium activated a current that was not significantly different to that observed in Na^+ containing external solution (Fig. 4B; $E_{\text{rev}}(\text{Cs}) -3.9 \pm 4.3$ mV; $n = 10$). These findings strengthen the view that hypotonic stretch-induced currents in retinal VSMCs are most compatible with the activation of TRPV2 rather than TRPP1 channels. To provide additional evidence of a role for TRPV2, the effects of tranilast (a TRPV2 inhibitor) on hypotonic stretch-induced currents in retinal VSMCs were examined. In the presence of tranilast, hypotonic stretch (recorded in Na^+ external solution) failed to elicit a significant increase in cation current activity (Fig. 4C). Overall, these results suggest that although retinal VSMCs express a range of putative mechanoTRP channels, only TRPV2 appears to underlie hypotonic stretch-induced cation currents in these cells.

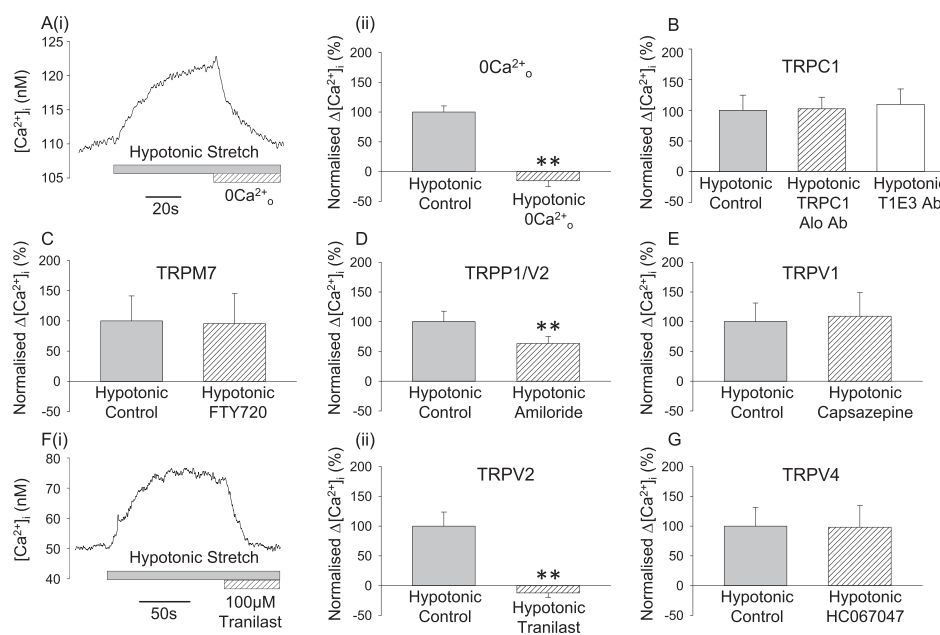


FIGURE 3. Effects of mechanoTRP channel inhibitors on hypotonic stretch induced Ca^{2+} influx in retinal VSMCs. **(A)** Representative trace **(i)** and mean data **(ii)** showing the rapid reversal of $[\text{Ca}^{2+}]_i$ responses evoked by hypotonic solution (Supplementary Table S3 in the online-only Data Supplement, solutions A1 versus A2) following removal of extracellular Ca^{2+} ($n = 6$). **(B)** Hypotonic-induced increases in $[\text{Ca}^{2+}]_i$ were unaffected by preincubating the vessels with TRPC1 pore-blocking antibody (T1E3 Ab; $n = 9$) or a control antibody targeting an internal epitope of the TRPC1 channel (Alo Ab; $n = 6$). Data were normalized to hypotonic responses in vessels not exposed to anti-TRPC1 antibody (hypotonic control; $n = 13$). **(C–G)** Effects of pharmacological inhibitors of TRPM7 **(C)**; FTY720, 1 μM ; $n = 5$), TRPP1/V2 **(D)**; amiloride; 100 μM ; $n = 8$), TRPV1 **(E)**; capsazepine; 5 μM ; $n = 6$), TRPV2 **(F)**; tranilast; 100 μM ; $n = 6$), and TRPV4 **(G)**; HC067047; 1 μM ; $n = 5$) channels. Significant inhibition of hypotonic stretch induced Ca^{2+} influx was only observed in the presence of the TRPP1/V2 antagonist, amiloride **(D)**, and the TRPV2 blocker, tranilast **(F)**.

TABLE 3. Summary of MechanoTRP Channel Protein Expression and Localization in Retinal VSMCs

MechanoTRP Channel	Plasma Membrane	Cytoplasm	Nucleus
TRPC1	+	++	+
TRPM7	+	+	+
TRPP1	+	++	+
TRPV1	+	++	+
TRPV2	++	+	+
TRPV4	+	+	++

+ and ++ indicate positive expression with ++ indicating higher levels.

TRPV2 Channels and Retinal Arteriolar Myogenic Signaling

A disadvantage of using hypotonic solution as a stretch stimulus is that plasma membrane ion channel activation may result not only from stretching of the plasma membrane but also from other osmotic effects.⁴⁵ Therefore, to confirm that TRPV2 channels could be activated by direct stretching of the plasma membrane, we used the cell-attached configuration of the patch-clamp technique. Suction was applied to the back of the patch pipette (−45 mm Hg) to stretch the membrane. Application of negative pressure produced around a 4-fold increase in transient cation current activity in retinal VSMCs. This persisted for the duration of the stretch and decayed when the pressure stimulus was removed (Fig. 5A). When tranilast was included in the patch pipette to inhibit TRPV2 channels, application of negative pressure failed to elicit any increase in stretch-activated cation current activity (Fig. 5B). To more specifically investigate the involvement of TRPV2 channels in stretch-activated cation currents in retinal VSMCs, we adopted a blocking antibody approach. When the patch pipettes were back-filled with a specific pore-blocking TRPV2 antibody raised against an extracellular epitope of the channel (1:100; Alomone ACC-039),⁴⁶ no significant increase in cation current activity was observed upon direct membrane stretch (Fig. 5C). As a negative control, we also tested the effects of a TRPV2 antibody targeted to an intracellular epitope of the channel (1:100; Millipore PC421). In the presence of this antibody, stretch-induced cation currents were still observed that were not significantly different to those recorded in normal control arterioles (Fig. 5D; $P > 0.05$). In separate experiments, membrane patches were exposed via the patch pipette to the TRPV2 agonist $\Delta 9$ -tetrahydrocannabinol ($\Delta 9$ -THC)⁴⁷ in the absence of any membrane stretch. Basal transient cation current activity was increased >20-fold compared to membrane patches not exposed to $\Delta 9$ -THC and resembled that evoked by stretching of the membrane (Fig. 5E). These findings provide further evidence that functional TRPV2 channels are present on the plasma membrane of retinal VSMCs and that these channels can be directly activated by membrane stretch.

To investigate the possible contribution of TRPV2 channels to the myogenic constriction of retinal arterioles directly, we carried out pressure myography experiments. Isolated arterioles were preincubated for 1 hour with pore-blocking or control TRPV2 antibodies and then cannulated and pressurized to 40 mm Hg to enable assessment of myogenic tone development. The development of myogenic tone was effectively abolished in vessels preincubated with TRPV2 pore-blocking antibodies (Fig. 6), demonstrating a requirement for TRPV2 in the myogenic response to pressure. In contrast, vessels exposed to the TRPV2 control antibodies developed a level of myogenic tone no different from that previously

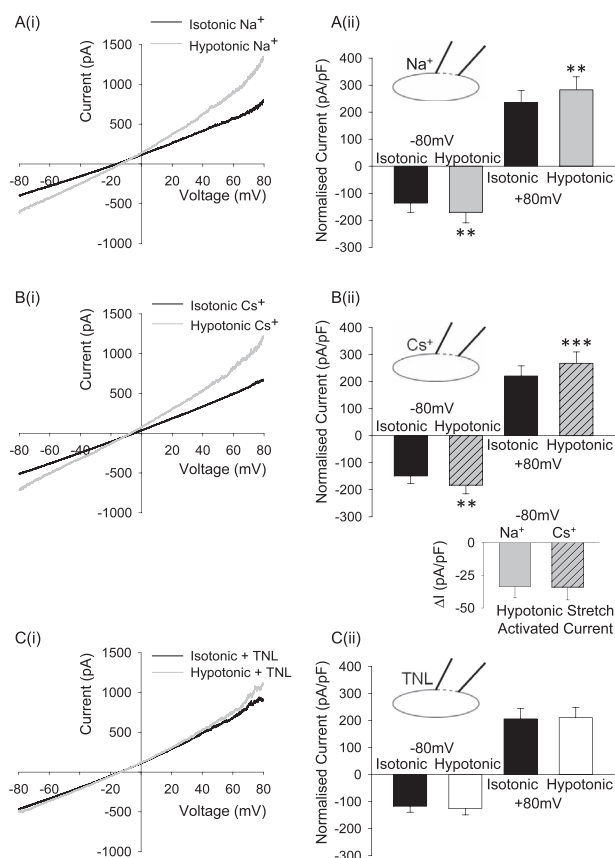


FIGURE 4. Hypotonic stretch-induced whole-cell cation currents are consistent with TRPV2 and not TRPP1 activation. **(Ai)** Representative whole-cell currents elicited by 1-second voltage ramps from −80 to +80 mV recorded first in isotonic and then in hypotonic Na^+ -containing external solutions (Supplementary Table S3 in the online-only Data Supplement, solutions A1 and A2). **(Aii)** Pooled data showing significant current activation by hypotonic solution at −80 and +80 mV ($n = 10$). **(Bi)** Equivalent whole-cell recordings **(i)** and summary data **(ii)** to those presented in **A**, but using isotonic and hypotonic external solutions where Na^+ was substituted by Cs^+ (Supplementary Table S3 in the online-only Data Supplement, solutions A3 and A4; $n = 10$ in **Bii**). Inset in **Bii**, summary bar chart showing that the amplitude of the hypotonic-induced current (difference current between isotonic and hypotonic conditions) at −80 mV did not differ significantly regardless of whether Na^+ or Cs^+ were used as charge carriers. **(Ci)** Typical whole-cell current traces from voltage ramps elicited in Na^+ containing isotonic and hypotonic solutions in the presence of the TRPV2 antagonist, tranilast (TNI; 100 μM). **(Cii)** Mean data showing that in the presence of tranilast hypotonic solution failed to evoke significant current activation at −80 and +80 mV ($n = 8$).

reported for these vessels (Fig. 6).^{12,40} Once the myogenic constriction had fully developed and stabilized in vessels exposed to the TRPV2 control antibodies (~15 minutes after pressurization), tranilast was applied in order to examine the involvement of TRPV2 channels in setting basal levels of myogenic tone. Tranilast caused a significant dilation of these vessels (Fig. 6), further confirming an important functional role for TRPV2 channels in the pressure-induced constriction of retinal arterioles. Application of tranilast to vessels preincubated with the pore-blocking TRPV2 antibodies was without effect (Fig. 6). To further substantiate our findings with tranilast, we undertook separate experiments to confirm that this drug was not having any off-target effects on other known myogenic signaling components in these vessels. In particular, we have previously shown that both Ca^{2+} release from

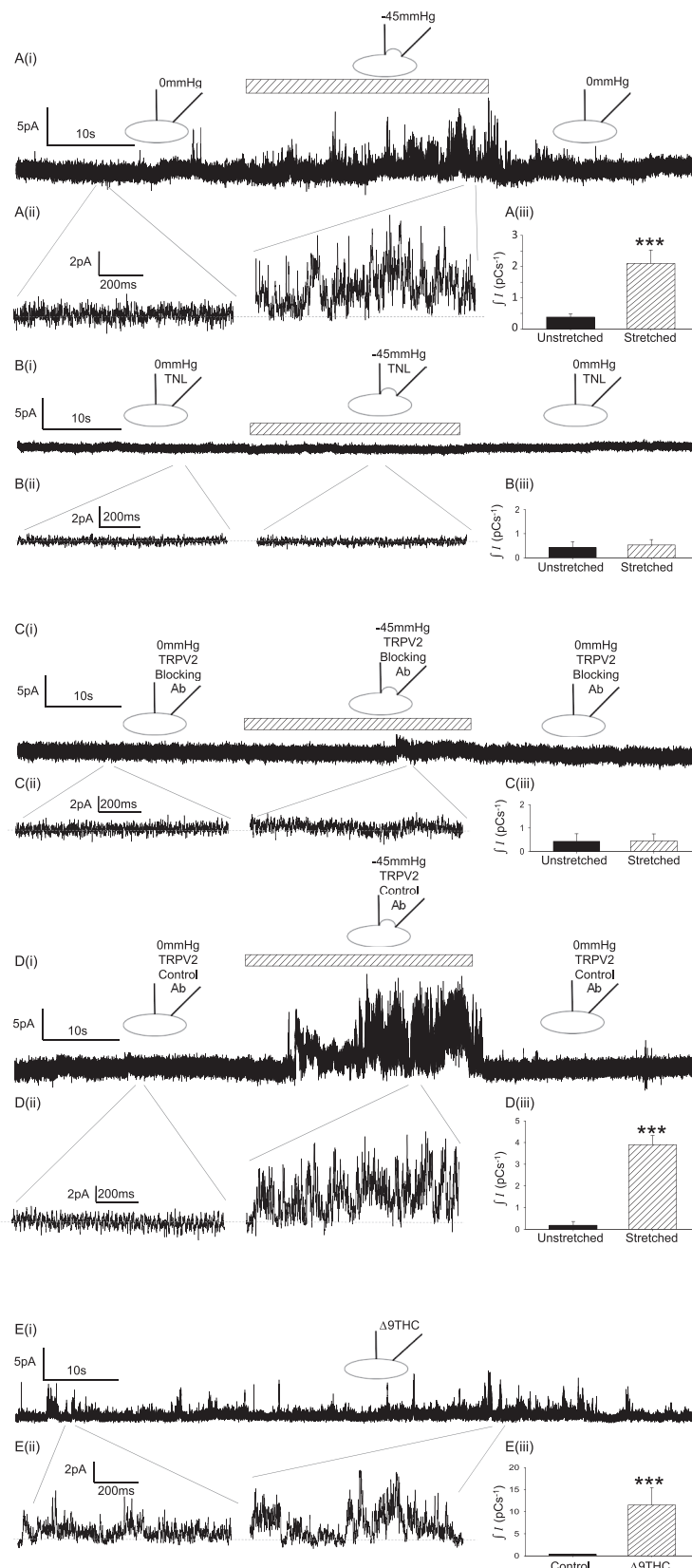


FIGURE 5. Mechanical stretch activates TRPV2-mediated cation currents. **(Ai)** Representative on-cell patch-clamp recording from a retinal VSMC showing changes in cation current activity as negative pressure (-45 mmHg) was applied to the back of the patch pipette. **(Aii)** Magnification of current traces in **Ai** showing stretch-activated cation current activity on a much faster time base. **(Aiii)** Summary data showing the mean integrated current density before and during membrane stretch ($n = 14$ patches). **(B)** Corresponding on-cell patch-clamp traces **(i, ii)** and summary data **(iii)** to those shown in **A**, but with the TRPV2 inhibitor, tranilast (100 μM), included in the patch pipette ($n = 10$ patches in **Bii**). Tranilast completely inhibited the induction of cation current activity during membrane stretch. **(C, D)** On-cell patch clamp measurements **(i, ii)** during direct membrane

stretch in the presence of pore-blocking (C; 1:100; Alomone ACC-039) or negative control (D; 1:100; Millipore PC421) anti-TRPV2 antibodies in the patch pipette. (C, Diii) Pooled data showing that the activation of stretch-activated cation currents was prevented by exposure to the pore-blocking ($n = 10$ patches), but not the negative control anti-TRPV2 antibodies ($n = 7$ patches). (E) Typical on-cell patch-clamp recording with the TRPV2 agonist, $\Delta 9$ THC (10 μ M), included in the patch pipette. (Eii) Expanded insets of $\Delta 9$ THC-stimulated cation current activity. (Eiii) Summary of the effects of $\Delta 9$ THC on current activity compared with control patches in the absence of this drug (control, $n = 19$ patches; $\Delta 9$ THC, $n = 9$ patches).

intracellular stores through ryanodine receptors (RyR) and Ca^{2+} influx via voltage-dependent Ca^{2+} channels are critically involved in the myogenic constriction of retinal arterioles.^{12,40} As shown in Supplementary Figure S2, tranilast had no effect on RyR-mediated Ca^{2+} release or voltage-dependent Ca^{2+} influx in these vessels as assessed using caffeine and high K^{+} solutions, respectively.

DISCUSSION

This study has provided clear evidence suggesting that TRPV2 is the principal mechanoTRP channel that underlies stretch-dependent cation current activity and myogenic vasoconstriction in retinal arterioles. Our results concur with previous studies suggesting that TRPV2 can act as a stretch sensor in VSMCs and contribute to cation currents and $[\text{Ca}^{2+}]_i$ rises evoked by hypotonic stimulation.²² They contrast, however, with studies in cerebral arteries, where TRPC6, TRPM4, and TRPP1 channels have been implicated as the major pathways

for stretch-dependent cation influx and myogenic vasoregulation.^{7,8,21} In this and previous work, we have been unable to detect TRPC6 and TRPM4 at the mRNA level in retinal arterioles (Fig. 1 and Ref. 24) and our electrophysiological data were inconsistent with the involvement of TRPP1 (PKD2) channels in mediating stretch-activated cation currents in retinal VSMCs (Fig. 4). These differences suggest that stretch-sensing mechanisms may vary in VSMCs of different vascular beds or in different segments of the circulation, given that we have studied microcirculatory vessels as opposed to larger resistance arteries.

We were surprised to find that VSMCs of retinal arterioles express numerous TRP channels previously implicated in mechanotransduction (Tables 2 and 3), yet only TRPV2 appeared to contribute to hypotonic stretch-induced Ca^{2+} influx and cation currents in these cells. Perhaps under different experimental conditions such as hypoxia or under in vivo conditions, these other channels may also participate in mechanotransduction. Our results, however, are in agreement

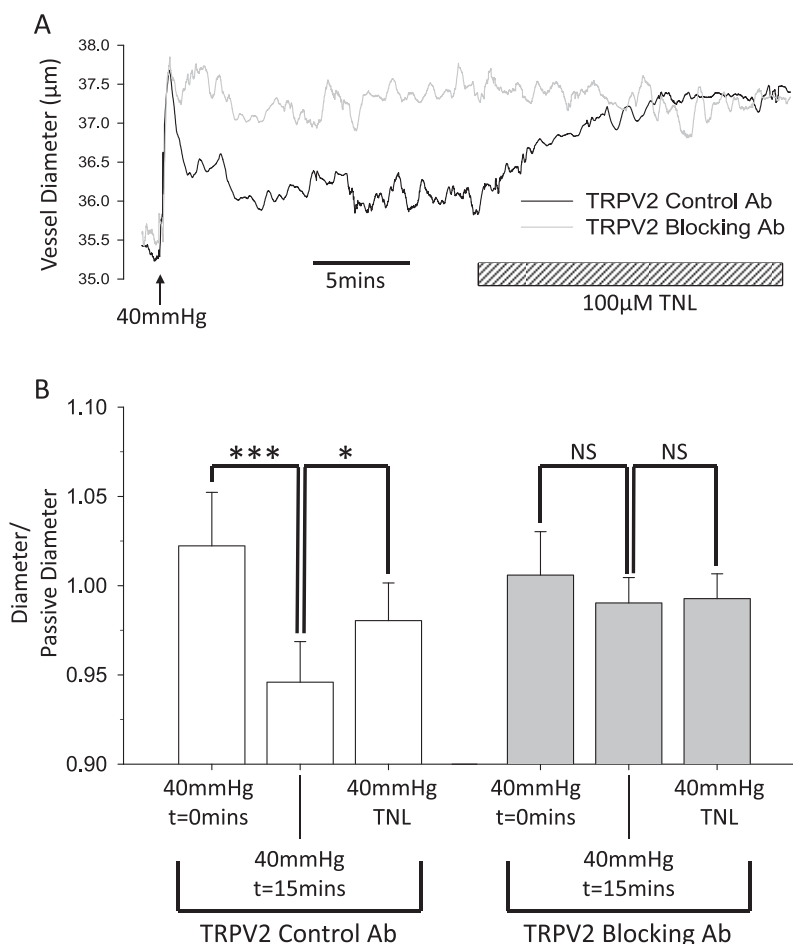


FIGURE 6. Effects of TRPV2 channel blockade on myogenic vasoconstriction. (A) Representative traces showing the effects of pore-blocking (1:100; Alomone ACC-039) and negative control (1:100; Millipore PC421) anti-TRPV2 antibodies on myogenic tone development in isolated retinal arterioles. The TRPV2 inhibitor, tranilast (100 μ M), was added 15 minutes after pressurization. (B) Mean diameter data (normalized to the maximum passive diameter) showing that the anti-TRPV2 pore-blocking antibodies abolished myogenic tone development and that tranilast caused a significant dilation of arterioles exposed to the negative control antibodies under conditions of steady-state myogenic tone ($n = 11$ for both groups).

with previous reports showing that although TRPC1 may be stretch-sensitive when expressed in heterologous systems,⁴⁸ data from TRPC1 knockout mice have indicated that these channels do not seem to contribute significantly to stretch-activated cation currents in VSMCs.⁴⁹ It is important to emphasize that many of the putative mechanoTRP channels that we have identified as being expressed in retinal VSMCs are known to be multimodal in their activation mechanisms and therefore could be playing other important functional roles. TRPC1, for example, has been proposed to act as a store-operated Ca^{2+} entry channel in some types of VSMCs,²⁸ while TRPM7 has been characterized as a constitutively active channel that plays a critical role in Mg^{2+} homeostasis in various tissues, including VSMCs.⁵⁰ In retinal arterioles, TRPP1 and TRPV1 were found to be mainly localized to cytosolic regions of the VSMCs, and it is therefore of relevance that both of these channels have previously been shown to reside on intracellular organelles and mediate Ca^{2+} release upon agonist stimulation.^{43,51} In cerebral VSMCs, endothelium-derived arachidonic acid metabolites (e.g., 11, 12 epoxyeicosatrienoic acid) have been identified as an important pathway for plasma membrane TRPV4 channel activation, which stimulates vaso-relaxation through a RyR/BK channel dependent mechanism.⁵² In retinal VSMCs, we found that the majority of the TRPV4 immunolabeling co-localized with the cell nuclei (Fig. 2G). Likewise, some co-localization of other TRP channels with VSMC nuclei was also apparent (Fig. 2; Table 3). Such findings are not completely unprecedented as we and others have previously reported nuclear co-localization of TRPV channels in various cell types including the vascular endothelium.^{38,53,54} Whether these channels play a functional role in the nucleus, however, remains unknown. Our current findings open up numerous new avenues for future research that could improve our understanding of the physiological function of TRP channels in the retinal microcirculation.

Our evidence that TRPV2 is the principal mechanoTRP channel involved in mediating stretch-dependent cation influx and myogenic vasoconstriction in retinal arterioles is supported by a number of observations: (1) TRPV2 message was detected in isolated retinal arterioles by RT-PCR; (2) confocal microscopy revealed strong plasma membrane localization of TRPV2 protein in retinal VSMCs; (3) hypotonic stretch-induced Ca^{2+} influx and cation currents displayed pharmacological and permeability properties consistent with TRPV2 (and not other potential mechanoTRP channels); (4) direct plasma membrane stretch during on-cell recording evoked transient cation current activity that could be blocked by inhibition of TRPV2 using tranilast or pore-blocking antibodies, and resembled that produced by the TRPV2 activator, Δ^9 -THC; and (5) TRPV2 pore-blocking antibodies prevented the development of myogenic tone in retinal arterioles and tranilast caused dilation of myogenically active vessels. Reconstitution of homotetrameric TRPV2 in liposomes has been reported to result in a channel with a conductance of 304 ± 4 pS using K^+ as the charge carrier.⁵⁵ In the present study, we were unable to definitively establish a single-channel conductance from our on-cell patch-clamp recordings due to the highly transient nature of the events and the absence of any obvious unitary current levels (Fig. 5). Nevertheless, the flickering behavior of the currents recorded, characterized by extremely short dwell times, correspond closely with the TRPV2 currents recorded in reconstituted liposomes and are almost identical to those attributed to TRPV2 channels elicited by stretch in aortic myocytes.²² Future studies using TRPV2 knockout animals will not only enable us to further explore the involvement of TRPV2 channels in myogenic signaling in the retinal vasculature, but also allow us to investigate their role in modulating blood flow autoregulation in vivo.

An important question that arises from this study is, how are retinal VSMC TRPV2 channels activated by stretching of the plasma membrane? Although the present study does not directly address this question, some useful insights may be gleaned from the existing literature. Recent work has suggested that TRPV2 channels may be activated by stretch via multiple mechanisms, including direct mechanical gating of the channel and stretch-induced translocation of TRPV2 from intracellular compartments to the plasma membrane through a phosphatidylinositol 3-kinase-dependent pathway.⁵⁶ In the present study, the speed of onset of stretch-induced TRPV2 channel activation in retinal VSMCs (3–10 seconds; see for example, Fig. 5) would appear most consistent with a direct mechanical gating mechanism, although stretch-induced trafficking of TRPV2 to the plasma membrane cannot be completely discounted. Another possibility is that plasma membrane bound TRPV2 channels in retinal VSMCs may not be directly mechanosensitive, but rather activated by one or more upstream signaling pathways that act to initially detect the stretch. In support of this idea, in cerebral VSMCs, opening of TRPM4 channels during stretch has been linked to the activation of a local signaling network involving AT_1 receptors, $\text{PLC}\gamma 1$, TRPC6, and Ca^{2+} release from IP_3 sensitive Ca^{2+} stores.⁵⁷ At present, little is known about the effects of intracellular signaling molecules on TRPV2 channel gating. Such knowledge represents an important starting point if we are to understand the possible involvement of upstream signaling pathways in the activation of retinal VSMC TRPV2 channels during stretch.

In conclusion, our data have demonstrated an important role for TRPV2 in the myogenic constriction of retinal arterioles. The work provides an important platform for better understanding the mechanisms involved in controlling retinal blood flow autoregulation in health and its disruption in disease. In the retina, loss of blood flow autoregulation is an early event in the pathogenesis of diseases such as diabetic retinopathy^{58,59} and glaucoma⁶⁰ and it will therefore be of interest in the future to investigate if changes in the expression or functional activity of TRPV2 channels contribute to the etiology of these diseases.

Acknowledgments

Supported by grants from the Biotechnology and Biological Sciences Research Council (BB/I026359/1) and British Heart Foundation (PG/11/94/29169).

Disclosure: **M.K. McGahon**, None; **J.A. Fernández**, None; **D.P. Dash**, None; **J. McKee**, None; **D.A. Simpson**, None; **A.V. Zholos**, None; **J.G. McGeown**, None; **T.M. Curtis**, None

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